EFFECTS OF NEUREGULIN-1 ON PERIPHERAL NERVE MYELINATION

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Introduction:

1 in 15 Americans has experienced peripheral nerve injury as a result of trauma or medical disorders[1]. While nerve tissue has the ability to regenerate naturally, the formation of scar tissue and an inflammatory response after injury can result in incomplete regrowth. Symptoms such as limb paralysis and chronic neuropathic pain due to cell death can be permanent[2].

In the event of a traumatic nerve injury, cell death is immediate. Axons and myelin degenerate 1-2 days after the injury in a process known as Wallerian degeneration[3]. Schwann cells then migrate to the site to begin the breakdown and phagocytosis of myelin and cell debris. After this step, they begin to proliferate as a support mechanism; this proliferation is essential for axonal regeneration. Schwann cells line the endoneurial tube in an elongated formation called bands of Bungner. They express myelin-associated proteins and neurotrophic factors to increase proliferation[4]. Studies show that axons respond chemotactically to these neurotrophic factors and grow in the direction of expression[5]. Therapies that make use of the Schwann cell's ability to guide growth through the protein expression gradient show promise for this reason. One of the factors expressed by Schwann cells, myelin-associated glycoprotein (MAG), has a few key roles in the regeneration pathway. In addition to myelinating regrowing axons, MAG is responsible for signaling macrophages to leave the area after the initial injury to reduce inflammation and allow for unimpeded growth. However, the high expression levels of MAG and other neurotrophic factors after injury are transient and decline sharply over the regeneration period[7]. Myelin Protein Zero (MP0) is also a key myelinating factor, especially in the case of adult axon regeneration. Because it is specifically involved in formation of the

myelin sheath, MP0 is usually upregulated after MAG begins initial myelination[7]. Schwann cells are the most common glial cell type in the peripheral nervous system and play an outsize role in regulating regeneration. Their denervation is a limiting factor for axonal regeneration and ultimately functional recovery, as well as a potential therapeutic target[2].

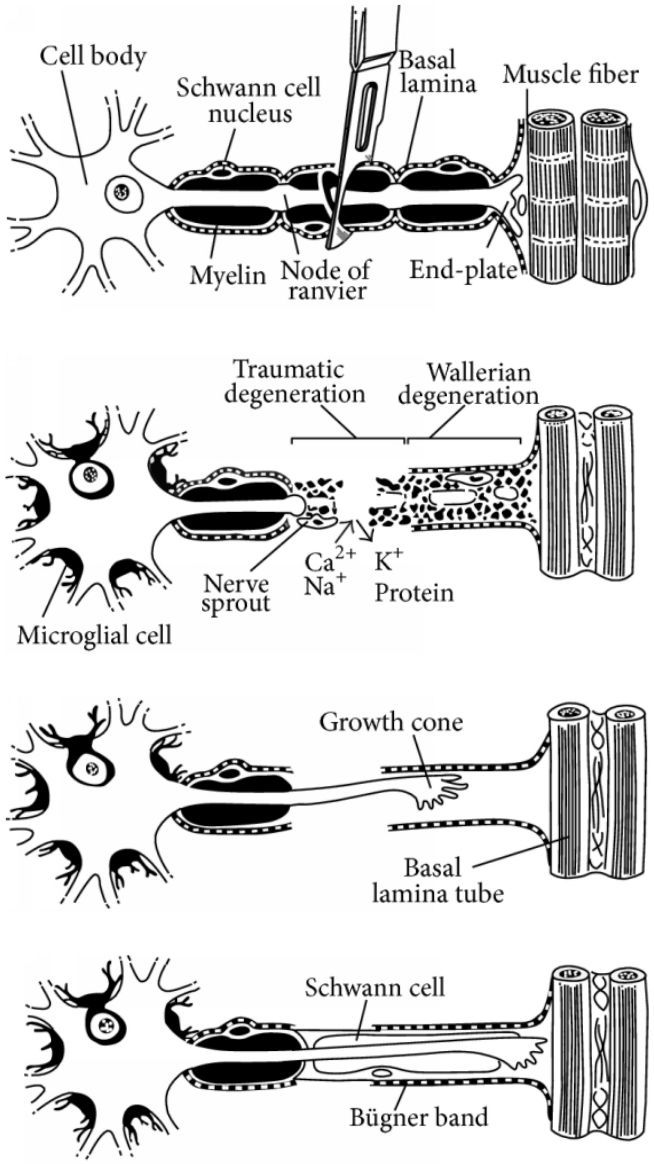

Figure 1. Regeneration process of neurons after a traumatic force injury. The initial Wallerian degeneration extends in both directions. Figure taken from St. Vincent's Hospital research study[1].

Without guidance across the nerve gap formed in peripheral nerve injuries, axons regrow disorganized, which can lead to eventual muscle atrophy. Ideally, the separated nerve endings are sutured together, but this cannot be done if the gap is too large and would cause significant tension on the nerve. The standard treatment for substantive nerve gaps involves grafting a nerve segment taken from a separate area in the patient's body, usually from expendable sensory nerves. While using the patient's own tissue (an autograft) prevents the immune response, the process requires multiple surgeries, and the donor site requires repair[3]. Furthermore, despite rapid advances in surgical methods, the nerves rarely

reach complete functional recovery[1]. Artificial scaffolds can serve as effective alternatives to nerve grafts. These scaffolds generally consist of an outer guidance channel and may also include elements within the channel to promote regeneration. The materials used to construct these scaffolds must have similar mechanical properties to nerves, prevent an immune response, and allow for oxygen diffusion in the area in addition to cell proliferation[8]. They must also be biodegradable and disappear when the area is healed in order to prevent pressure once the nerve has grown back.

Collagen, a protein that makes up most of the connective tissue in the body, is a promising candidate for various tissue engineering applications because of its biocompatibility[4]. To date, collagen is the only clinically approved biopolymer on the market for peripheral nerve regeneration. It is used for neuroregenerative therapies because it can support nerve regeneration at a damaged site and biodegrade when it is no longer needed, thereby avoiding an immune reaction[5]. In order to generate the mechanical strength needed to support nerve regeneration, collagen nanofibers can be created through electrospinning. Electrospinning uses electrical energy to create thin nanofibers out of a solution. This process enhances the mechanical properties of the original material, and the resulting fibers are porous, allowing for diffusion as well as the addition of growth factor to the material[3]. These fibers also create a 3-dimensional environment that resembles the native extracellular matrix of the peripheral nerve microenvironment. The high surface area of the fibers has been linked to better cellular attachment. Because of this, electrospun scaffolds demonstrate significant functional recovery. Previous studies show that Schwann cells in particular adhere well to and elongate along electrospun fibers, forming structures similar to bands of Bungner. The fiber

orientation can also guide growth in a certain direction, for example from the proximal to the distal nerve ending. Furthermore, electrospinning allows better control over material properties such as the porosity and degradation rate. This is done by altering the solution concentration and potentially the ratio if the material in question is a composite. As a natural polymer, collagen has a rapid degradation rate, so mixing it with a synthetic polymer to create a composite can help with stability[9]. The first 10-12 weeks after injury are crucial for axonal regeneration and functional recovery, so the conduit should only begin degradation after that point.

Polymeric nanoparticles protect therapeutic factors and deliver them efficiently to the target site. Poly(lactic-co-glycolic acid) is a composite made of two polymers: polylactic acid, or PLA, and poly(glycolic acid), or PGA. PLGA degrades slowly using a hydrolysis mechanism, meaning these microparticles are also highly biodegradable. The precise degradation rate of the material as well as other characteristics like permeability can be controlled by altering the PGA:PLA ratio. Polymeric microparticles are often used as drug carriers because of their efficiency and biocompatibility. PLGA microspheres in particular can prevent drug degradation, prolong release, and reduce toxicity[10].

Another extremely biocompatible polymer used extensively in peripheral nerve regeneration research is alginate. As a natural biopolymer, alginate mimics the structure of the extracellular matrix (ECM) leading to low toxicity, but it also very quickly degrades. This can be remedied using a number of modifications, most notably by chemically coupling Arg-Gly-Asp (RGD) peptides, forming RGD-alginate in the process[8]. This material has been used in

drug and immune cell targeting in the past, and shows promise for microparticle drug delivery in the context of the peripheral nervous system.

A potential growth factor to be used in conjunction with the nerve conduit is Neuregulin 1 (NRG1), a protein involved in signaling for the repair of damaged nerves. While this protein has many different isoforms with varying capabilities, this study will focus on NRG1 Type III, also known as Sensory and Motor-neuron Derived Factor (SMDF). SMDF binds to Schwann cell receptors and kicks off a signal transduction pathway that promotes myelination, the formation of a sheath around the axons in a nerve. Myelin sheaths protect the axon and have been shown to increase conduction velocity in regenerating nerves[11]. SMDF is normally a transmembrane protein, but it can be delivered as a soluble protein and trigger the same increase in myelin expression. However, this reaction is dose-dependent, so controlled release from the conduit and the effect of dosage on nerve cells must be elucidated in order to assess the effects of SMDF within the peripheral nervous system. The incorporation of a drug delivery mechanism into a nerve conduit such as the microparticles mentioned above would allow for extended drug release over time. The effects of SMDF can be compared to other neurotrophic factors in order to accurately assess the efficacy of this potential new drug candidate. Nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) are two proteins that have been previously used in experimental trials for peripheral nerve regeneration. Both are expressed naturally by Schwann cells after injury. NGF has been proven to prevent axonal degeneration and GDNF promotes myelination[5]. Based on preliminary studies, incorporating SMDF into electrospun collagen nerve guidance channels will result in enhanced cell and tissue growth as well as improved functional recovery.

Determining the efficacy of NRG1 in the nerve repair process is crucial to further elucidating the pathways necessary for tissue regeneration as well as potentially providing a better therapeutic avenue for peripheral nerve injuries.

Methodology and Expected Outcomes:

The in vivo experiments conducted focus on the effects of SMDF directly applied to Schwann cells, the nerve cells responsible for much of the regeneration response. Cell culture was performed on S16 cells, an immortalized Schwann cell line taken from the sciatic nerve in rats. S16 cells resemble early Schwann cells as they prepare to myelinate[7]. The cells were cultured under standard cell culture conditions (a humidified, 37°C, 5% CO2/95% air environment) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin−streptomycin solution. Cells were passaged into 96-well plates at a density of 10,000 cells per well to perform drug dosage experiments. This included both flat-bottomed plates treated with laminin, a key nervous system ECM protein, as well as plates precoated with aligned poly-L-lactic acid fibers for 3D cell culture (Mimetix). They were then dosed with 1, 10, and 100 ng/ml of SMDF in addition to complete culture media. In addition, there was a negative control group receiving media without any growth factor and two positive control groups receiving 1, 10, and 100 ng/ml of Nerve Growth Factor (NGF) and Glial cell line-Derived Neurotrophic Factor (GDNF). The experiment was performed in triplicate and the media was replenished daily. After 3 days, the cells were fixed in 10% formalin.

The fixed cells underwent immunostaining for identification of myelin-associated factors Myelin Protein Zero (MP0) and MAG. Cell imaging was performed using the Cytation 5 and

fluorescence of each well was quantified. This experiment was essential for determining the optimal dosage of SMDF that stimulates significant myelination in Schwann cells. Presumably, myelination will increase with dosage up to a certain point at which high dosages will start inhibiting myelination.

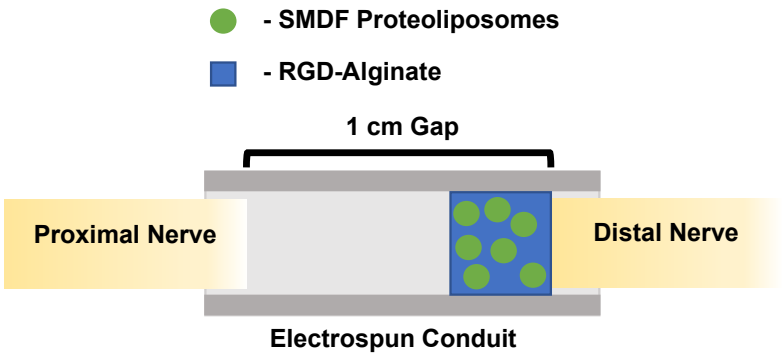

Figure 2. The conduit, consisting of electrospun collagen, contains microspheres containing SMDF, which allows for controlled release of growth factor over time. These microspheres are situated within alginate hydrogel to prevent immediate release of SMDF. This figure shows the hypothetical placement of the conduit at the site of a nerve gap.

After determining the best dosage, SMDF was loaded into microspheres, mixed with alginate hydrogel, and then incorporated into a nerve conduit (Fig. 2). This conduit was primarily composed of collagen along with poly-ε-caprolactone (PCL) to provide more mechanical strength and longer degradation time, both from Sigma-Aldrich. Collagen was lyophilized and then solubilized in HFP using previously detailed methods, after which electrospinning occurred[9]. Electrospun fiber diameters were 1 um in cross-sectional diameter, a standard size that was produced by altering concentration of collagen and PCL during electrospinning. Formation of PLGA microspheres and RGD-alginate hydrogel were formed using previously detailed methods[10]. To analyze growth factor release over time, the drug-loaded conduit would be submerged in phosphate-buffered saline (PBS) and placed on a rotary shaker at 37°C, mimicking body temperature. The amount of drug released into solution would be quantified at 1, 3, 7, 14, 21, and 28 days using an enzyme-linked

immunosorbent assay (ELISA) designed to detect SMDF. The biodegradability of the conduit within the body can be estimated by placing the conduits in an aqueous PBS solution containing 0.1% collagenase. The time taken to fully degrade the collagen conduit both with and without SMDF will be recorded. The drug release profile is expected to be a logarithmic curve, with an initial "burst" of growth factor release after the first day preceding a gradual decrease in growth factor released over time. The degradation rate is expected to be comparable to the release rate; ideally, the conduit fully degrades only when there is little to no SMDF left in the device. In the future *in vivo* studies would be conducted to view the effects of the device in an animal model.

Results:

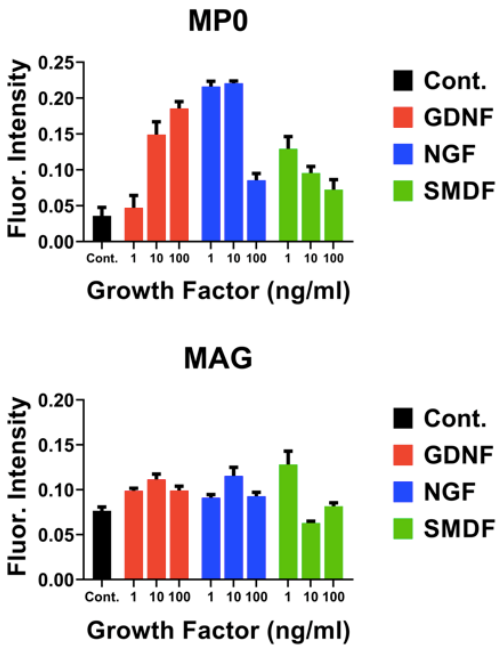

Figure 3. Myelination increases due to SMDF are comparable to standard nerve growth factors. Staining intensity was analyzed in single cells to compare myelination density between groups. Two different myelin markers were used; MAG+ staining indicates the development of a myelin sheath while MP0+ is only present in fully formed myelin sheaths.

Figure 3 shows data collected from the 96-well flat plate trial. When levels of Myelin Protein Zero (MP0) and Myelin Associated Glycoprotein (MAG) are quantified through cell imaging, cells treated with each growth factor show comparable myelination. The negative controls show lower myelination across the board. GDNF shows increasing myelination with increasing concentration. NGF shows some decrease in myelin expression at concentrations of 100 ng/ml, indicating some cytotoxicity at this high dosage. The experimental SMDF groups

show increased myelination over the negative control, but they also show gradually decreasing myelination as concentration of the growth factor increases. This may imply some toxicity at higher levels, similar to NGF.

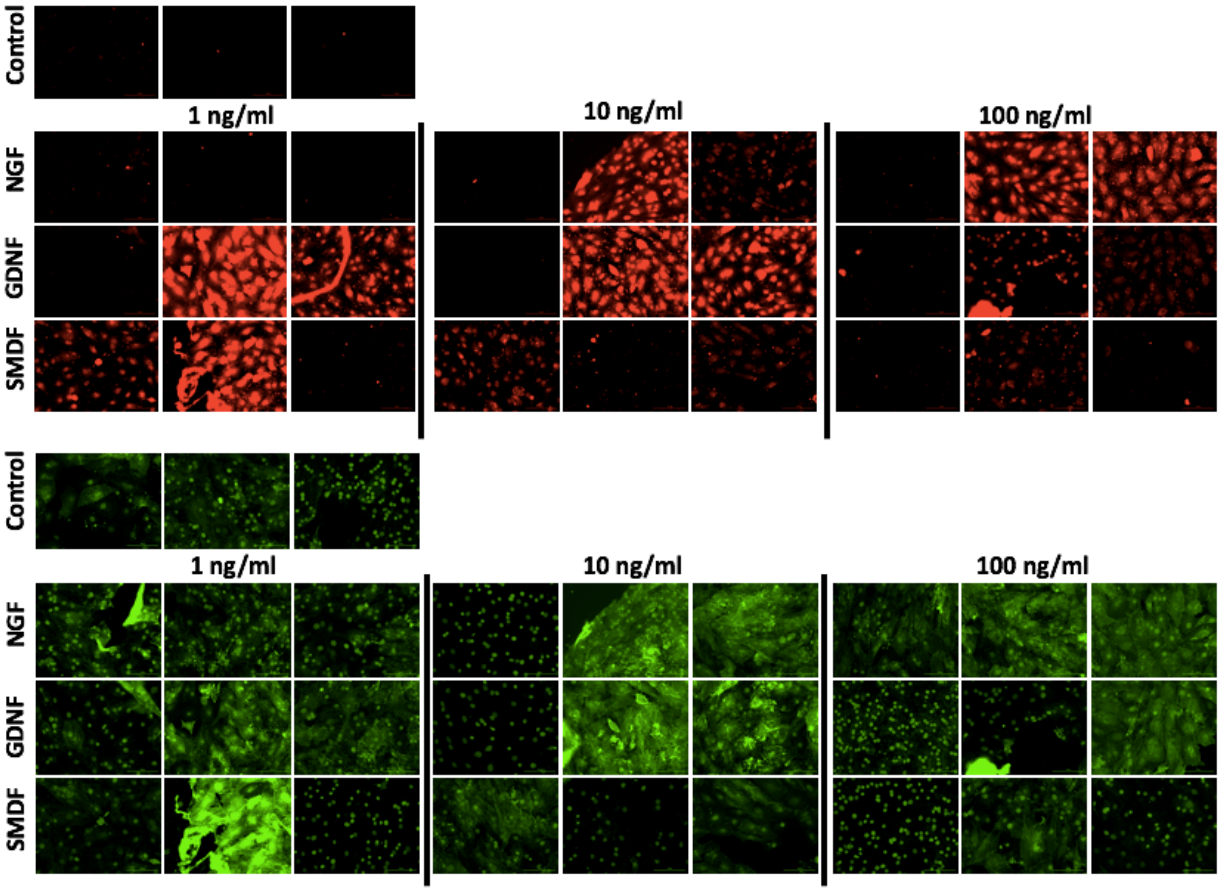

Figure 4. Fluorescent microscopy images of Schwann cells on flat 96-well plates (n=3) illustrate significant myelination. Cells underwent four different treatments: a control group with no growth factor treatment; NGF, a growth factor involved in many different nerve regulatory pathways; GDNF, which promotes neuronal cell survival; and SMDF, the protein of interest. Within these treatment groups, each growth factor was administered in trials of 1, 10, and 100 ng/ml to visualize effects dependent on drug dosage. Primary staining was conducted with anti-myelin antibodies, and secondary staining was conducted using fluorescent antibodies.

Looking at the microscopy images in Figure 4 further elucidates the mechanisms that may be occurring. Regardless of treatment, some wells have much higher myelin expression levels than others in the same experimental group. These results may imply that while growth factors aid in myelin expression, the cells themselves eventually start signaling to each other once they reach a certain myelin threshold, after which all cells in the area start expressing

high levels of myelin. Future therapies and further research should focus on identifying this threshold and the factors that encourage cells to meet it. Overall, SMDF seems to show the most consistent (though not the highest) levels of expression compared to the positive and negative controls.

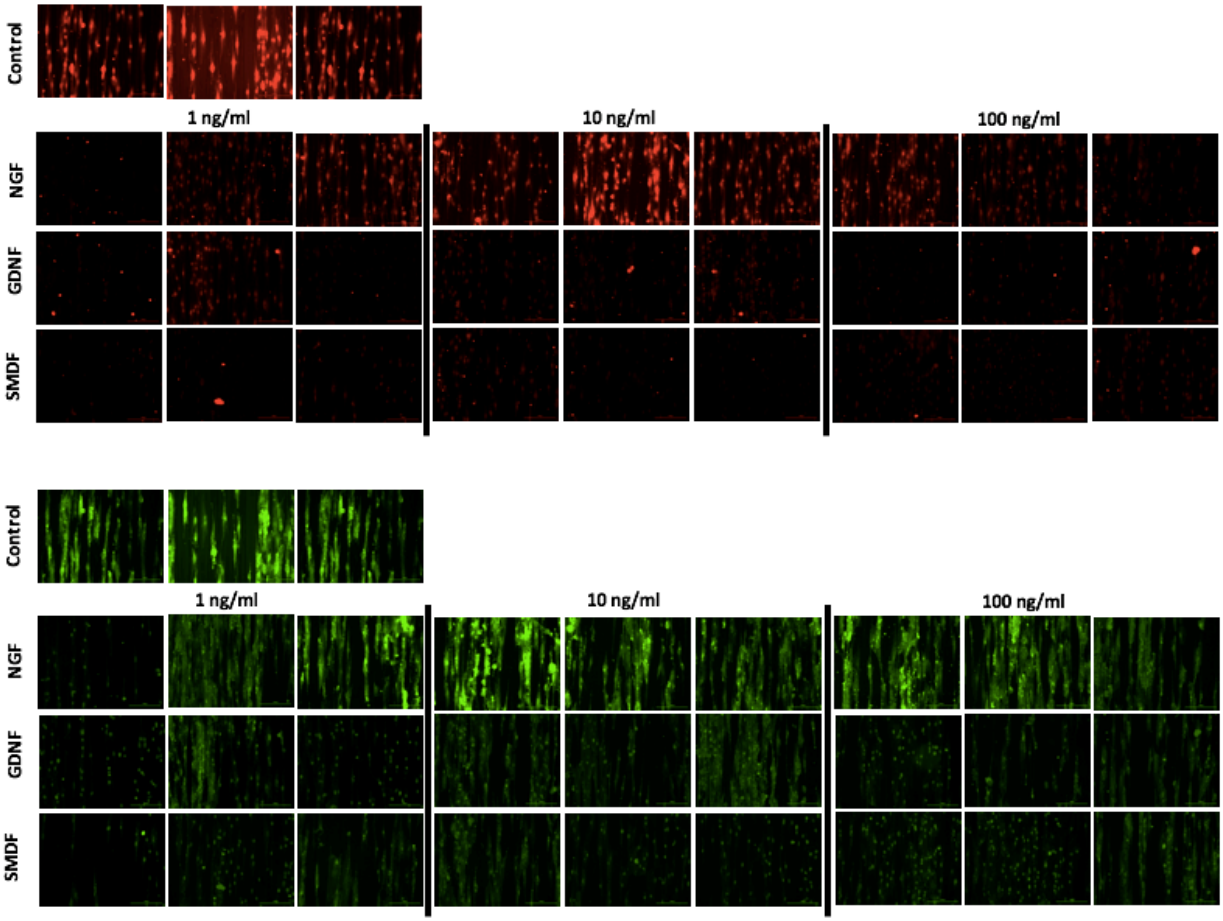

Figure 5. Fluorescent microscopy images of Schwann cells on fiber plate 96-well plates. The experimental groups, dosages, and primary antibodies used were the same as in Figure 3. The unique fiber alignment of the wells illustrate the natural arrangement of Schwann cells in bands of Bungner.

Interestingly, the experiments conducted on the fiber plates (Fig. 5) show significantly more variability in their results. The formation of the bands of Bungner is clear as the cells formed aligned myelin sheaths. However, there appeared to be no significant difference in myelin expression across the board, as even the positive controls showed less myelination than the negative control.

Discussion and Conclusion:

Peripheral nerve regeneration involves multiple cellular pathways. Schwann cells especially play an important role as the key instigators of phagocytosis of myelin debris and remyelination. Optimizing nerve guidance channels by incorporating drugs that target Schwann cells—in this case SMDF—is a novel way to improve outcomes after traumatic injury.

Cell culture and immunostaining methods indicated a significant increase in myelination after treatment with SMDF over the negative control. While the positive controls (NGF and GDNF) primarily served to confirm the success of the assays, SMDF showed comparable results with respect to the positive controls regardless. This implies promise in using SMDF as a potential drug candidate in further peripheral nerve regeneration experiments.

In terms of testing SMDF on fiber plates, results were mixed. This may be due to the fundamental difference in structure of the flat and fiber plate assays. Fiber plates allowed for formation of thin elongated myelin sheaths, very similar to the myelination process *in vivo*. Because this 3D morphology was not able to arise on the flat plate assays, this may have resulted in continuous upregulation of myelin-associated factors and therefore a brighter signal. Schwann cells may perform different processes on fiber plates with different myelination cues. Once the correct 3D morphology has been formed, there is no longer any need to upregulate myelination. Further trials must be conducted to determine the true mechanisms behind myelination in both 3D and 2D cell culture.

In addition to repetition of the flat and fiber plate experiments, nerve conduit analysis must be performed in order to ascertain the optimal composition and drug concentration for remyelination *in vivo*. This involves biodegradability assays and controlled release experiments

(ex. ELISA). As mentioned before, the most integral nerve regeneration occurs up to 10-12 weeks after peripheral nerve injury, meaning that an effective conduit must last at least 10 weeks before beginning to degrade[1]. The degradation rate is also essential to determine, as the conduit must degrade quickly enough to avoid blocking the final stages of axon regrowth, which would necessitate a second surgery. Controlled release trials must also last 10-14 weeks, as the sharp dropoff in myelin regenerating factors is ultimately what leads to incomplete regeneration in clinical cases. These experiments would eventually give way to *in vivo* experiments, a common one being severing the sciatic nerve in rat models and implanting the drug delivery conduit over a period of weeks to months. This treatment would be compared to a conduit implanted without growth factor to determine the effects on regeneration through immunostaining, as well as functional recovery over time (carried out using behavioral tests and electromyogram analysis).

Peripheral nervous system injuries drastically decrease patient quality of life, often leading to chronic pain and disability. Current treatment methods fall short of basic functional recovery standards, sometimes leaving patients paralyzed for life[1]. Mitigating morbidity and lifelong disability is crucial to advancing the field of peripheral nerve regeneration, and carries implications for regeneration within the central nervous system as well. We must aim to discover more reliable treatments beyond autografts to further advance the fields of medicine and neuroscience.

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